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Research Article

BIOPROSPECTING OF SOME ETHNOMEDICINAL PLANTS FOR POTENTIAL ANTIMYCOBACTERIAL BACTERIOCIN LIKE INHIBITORY SUBSTANCES (BLIS)

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ABSTRACT: Microbes produce antimicrobial substances giving itself an advantage to compete for resources over other microbes. In this study, some ethnomedicinal plants were screened for microbes that can produce anti-mycobacterial bacteriocin and have isolated a microbe. The isolated microbe has morphological characteristics of Gram positive and endospore forming rods. The 16S rRNA molecular level sequencing shows a 100% similarity index with *Bacillus subtilis* of GenBank sequences. The bioactive compound is a peptide like substance exhibiting a clear zone of inhibition with *Mycobacterium smegmatis* as indicator microbe. Being sensitive to proteases, confirms its proteinaceous nature, shows characteristics of heat stability up to 70°C and extractability with wide range of organic solvents. Having an advantage of being heat stable, tolerant to various organic solvents, and (MIC) minimum inhibitory concentration of 400 µg /ml for an unoptimized bacterial isolate, this compound might have pharmacological significance.

Key words: Anti-mycobacterial drugs, *Bacillus subtilis*, BLIS, Microbiome, Minimal Inhibitory Concentration.

INTRODUCTION

Many bacteria are capable of producing can antimicrobial substances, using such antimicrobial substances like antibiotics are important pillars of modern medicine (Ball *et al*. 2004). All these Antibiotics and other antimicrobial substances, over a period, lose their efficacy against pathogenic bacteria and might develop resistance (Ventola 2015). The unprecedented rise in the antimicrobial resistance (AMR) becomes a global concern (WHO 2018). Some of the reasons could be the overuse or abuse of antibiotics. Treatment of bacterial infections resistant to first-line drugs can be toxic and expensive (Sotgiu *et al*. 2015). Hence, timely replacement with new and potential antimicrobial substances is inevitable to combat the pathogenic bacteria (Lancet 2009).

Mycobacterium tuberculosis causes Tuberculosis (TB) and its resistance has become a global burden as it ranks alongside HIV as a leading cause of death worldwide. In 2014, 6 million new cases of TB were reported to WHO, a total of 1.1 Lakh people started MDR-TB [multi-drug resistant TB] treatment in 2014, an increase of 14% compared with 2013. Extensively drug-resistant TB (XDR-TB) had been reported by 105 countries by 2015. An estimated 9.7% of people with MDR-TB have XDR-TB. Statistical rise of Total-drug resistance TB and TB resistant to first-line drug treatment are alarming. Therefore, new potential drugs and vaccines will be required to achieve the targets set in 'The End TB Strategy' by WHO. (Tiberi *et al*. 2018).

Many studies have indicated that microbes may interact with medicinal plants to alter their phytochemical production influencing their efficacy and may themselves be capable of producing chemical agents or antibiotics that can combat the pathogens (Huang *et al*. 2018, Marcel *et al*. 2016). India, having a diverse ethnobotanical life with rich microbiome of immense value, has long been using medicinal plants for the treatment of several diseases and still follows pluralistic healthcare system with more than 70% of its 1.1 billion population using

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non-allopathic systems of medicine (Ashok *et al*. 2007). Plant-associated microbes pose an attractive and promising source for producing such antimicrobial substances and are nearly unexplored (Beattie 2015, Zasloff 2003). Therefore, a great deal of current research is focused on bioprospecting of plant microbiomes, as they have highly specialized and co-evolved genetic pool with rich secondary metabolism (Backer *et al*. 2018).

The present study focuses on bioprospecting Indian ethnomedicinal plants for their microbiomes that can produce bacteriocins that are antimycobacterial in nature with *M. smegmatis* as the indicator organism.

MATERIALS AND METHODS

All chemicals were purchased from SD Fine-Chem Ltd (India), trypsin (T- 8918; Sigma), proteinase K (P- 2308; Sigma), polyether sulfone membrane syringe filter, pore size 0.22 µm (Merck), Soxhlet apparatus, rotatory evaporator (Rotavapor® BUCHI Labortechnik AG), lyophilizer (Scientek Services), *Mycobacterium smegmatis* MC^2 -155 wild type .

Sample collection and preparation

Fresh, mature and healthy leaves of *Ficus benghalensis*, *Ficus religiosa*, *Ficus starlight*, *Ficus benjamina*, *Ficus krishnae*, *Saussurea obvallata*, *Curcuma aromatica*, *Tinospora cordifolia* and *Leucas aspera* were collected from different regions of North and South Bengaluru (2016 - 2018), India. The leaves were washed, shade dried, powdered and stored in dry air-tight containers in dark place. 10 grams of stored powdered leaf samples were used for methanolic extraction by the Soxhlet apparatus. The extracts were concentrated using rotatory evaporator and used for screening experiments.

Indicator bacteria

M. smegmatis was taken as an indicator organism*;* it is a non-pathogenic bacterium and an effective model organism for tuberculosis drug discovery (Altaf *et al*. 2010). *M. smegmatis* (MC^2-155 wild type) was cultivated at 37°C for 48 h in standard Lysogeny Broth (LB) with tween 80. Turbidity of the cultures was matched with that 0.5 McFarland standard and was used for all the experiments.

Screening of bacteriocin producer

Methanolic plant extracts were diluted using DMSO (dimethyl sulfoxide) at dilutions of 1 mg/ml, 5 mg/ml and 10 mg/ml for screening. Spread plate and Spot-on-lawn method were used for screening the antimicrobial agent producing microbes (Tagg *et al*. 1971). 30 µl of each of these aliquots were loaded into 6 mm wide wells made in LB agar plates pre-swabbed with indicator organism. The plates were incubated at 37°C for 24 h and were checked for bacteriocin producing organism. Antagonistic activity of bacteriocin producer was reconfirmed by doing soft agar over lay technique (Gratia 1936) and isolates that showed better zone of inhibition (ZOI) as a measure of antagonism, were selected for identification.

Identification and characterization of bacteriocin producer

Isolate that showed maximum inhibitory zone, was preliminarily identified through their morphological features and followed by Gram's staining and biochemical tests like IMViC (Indole, methyl red, Voges-Proskauer and citrate), hydrolysis of starch, lipid and gelatin, carbohydrate fermentation tests for lactose, fructose and galactose. Further, the isolate was characterized by 16S rRNA sequencing. Using specific primers (Table 1), the genomic DNA fragment was amplified by Peltier Thermal Cycler (PTC-225) under specific conditions for 30 cycles. The obtained sequence was aligned by using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1997) and phylogenetic tree was constructed.

Determination of antimycobacterial activity

The selected bacterial isolate was cultured in standard liquid media at 37°C for 24 h under agitation. Cultured broth was centrifuged for 15 min at 8000 rpm and the supernatant was concentrated using lyophilizer. The concentrated supernatant was extracted with equal volumes of various organic solvents like ethyl acetate, butanol, acetone, acetonitrile, methanol, propanol and chloroform based on their affinity towards organic solvents (Burianek *et al.* 2000). Butanol was used as third solvent in polar solvent extractions, in variation to dialysis of interfacial solvents. The solvent mixtures were thoroughly agitated at 160 rpm for an hour and the separated organic fractions were left for evaporation at 45°C, dried residues were dissolved in 1 ml of sterile DMSO [Dimethyl sulfoxide] followed by ultra-filtration with membrane filters of pore size 0.22 µm to control endospores. To evaluate the antimycobacterial activity of the obtained extracts with indicator organism, well diffusion assays were carried out in quadruples (Balouiri *et al.* 2016). For this study, 30 µl of each of dried organic solvent extracts dissolved in DMSO, were loaded into 6 mm wide wells made in LB agar plates pre-swabbed with 50 µl indicator inoculum, then plates were incubated at

Table 1. Primers used in the experiments.

Primer Name	Sequence Details	Number of Base
27F	5' AGAGTTTGATCMTGGCTCAG 3'	20
1492R	5' TACGGYTACCTTGTTACGACTT 3'	20

Table 2. Biochemical characteristics of isolate-1.

Test		Result
Starch Hydrolysis		+
Lipid Hydrolysis		+
Gelatin Hydrolysis		+
Indole test		-
Methyl Red test		-
Voges-Proskauer test		-
Citrate Utilization test		+
Carbohydrate fermentation	Lactose	-
	Fructose	+
	Glucose	+

Table 3. Effect of heat on the activity of BLIS.

Temperature (°C)	Antimycobacterial activity (ZOI-mm)	Relative activity (%)
Control (35)	15	100
40	15	100
50	15	100
60	15	100
70	15	100
80	12	80
90	10	67
100	9	40
121	No inhibition	-

37°C for 24 h and were checked for zone of inhibition. The dried organic solvent extract, that showed maximum zone of inhibition with the indicator organism as a measure of antimycobacterial activity was selected for further study.

Effect of proteases and heat

To study the effect of proteases, 0.25 ml of extracts were separately added with 1.25 mg of Trypsin and Proteinase K and sample without proteases was taken as control. All the samples were incubated at 37°C for 24 h. Thermal stability of the sample was examined by incubating samples for 10 min under moist heat treatment ranging from 40°C to 121°C by 10°C rise. Samples unexposed to heat and proteases were taken as control. All the treated samples were tested for antimicrobial activity.

Determination of Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of the butanolic extract was determined by broth dilution method using sterile LB tween broth as the test media (Roberts *et al.* 2014). To 1 ml of test media taken in different test tubes, dried butanolic extract prepared in a series of two-fold dilution with DMSO ranging from 100 µg to 1000 µg/ml was added and 1 ml of DMSO alone with test media acted as control. Tubes were added with 100 µl of *M. smegmatis* inoculum (OD – 0.6 nm) and incubated for 24 h at 37°C under sterile conditions. The lowest concentration of the bacterial isolate that did not give any significant growth is taken as the Minimum inhibitory concentration.

RESULTS AND DISCUSSION

Screening of ethnomedicinal plant samples

A total of nine isolates were obtained on spread plate and spot-on-lawn assays, showing positive association, partial and clear inhibition with *M. smegmatis* as an indicator organism. Isolate-1 from *F. benghalensis* showed full inhibition exhibiting clear antagonism and competitive inhibitory effect on the indicator organism (Fig. 1a), isolate 9 from *F. religiosa* showed partial inhibition, isolates 2 to 7 from *F. benjamina* and 8 from *F. krishnae* exhibited positive association with the indicator organism. Samples from *C. aromatica, F. starlight, S. obvallata, T. cordifolia* and *L. aspera* did not show any growth in response to the indicator organism. Antagonistic activity of bacterial isolate-1 was further reaffirmed with the soft agar overlay assay (Fig. 1b).

Identification and characterization of Bacterial isolate

The selected isolate-1 was identified as bacterium based on their morphological and biochemical characteristics. Isolate-1 showed characteristics of gram positive, spore forming rods and colony morphology showed slimy, flat and irregular colonies with lobate margins (Fig. 2). On biochemical characterization, isolate-1 showed positive for citrate utilization, starch, lipid and gelatin hydrolysis while negative for IMV tests.

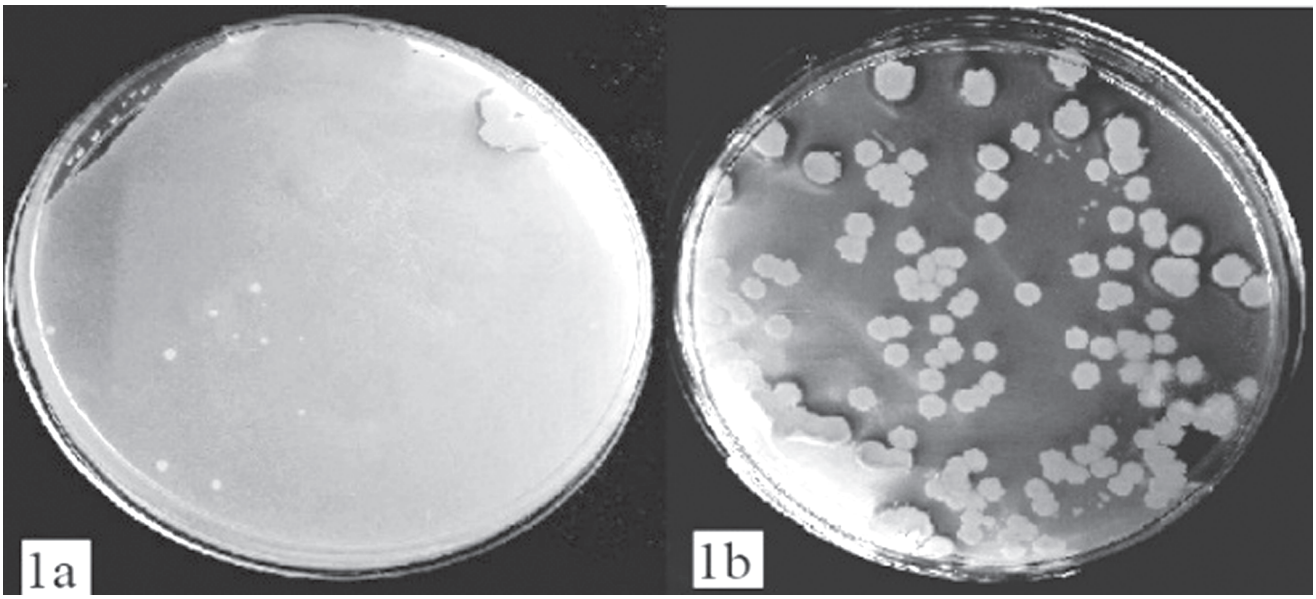

Fig. 1. (a) Isolate-1 showing antagonistic effect with indicator organism; (b) Soft agar overlay assay of isolate-1.

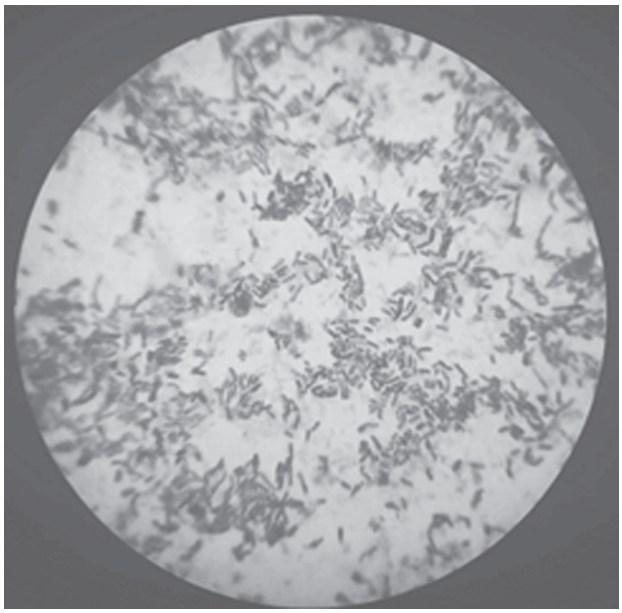

Fig. 2. Gram staining of Isolate-1 shows gram-positive rods with few endospores.

Carbohydrate fermentation tests were positive for fructose and glucose but negative for lactose (Table 2). BLAST results of 16S rRNA gene sequence confirms that the bacterial isolate belongs to the genus *Bacillus* with highest identity to *B. subtilis* (Fig. 3) with the accession number of MK733983 in GenBank nucleotide sequence database.

Antimycobacterial activity of the bacterial isolate

The crude culture supernatant fraction showed inhibitory zones with indicator organism, specifying that the antimycobacterial compound produced by isolate 1. The results of the well diffusion assay show the capability of tolerance to various organic solvents. Extraction with butanol showed a highest ZOI of 22 mm, followed by extraction with ethyl acetate (14 mm), chloroform (12 mm), acetonitrile (12 mm), acetone (10 mm) while extraction with propanol and methanol did not show any activity (Fig. 4a and 4b).

Effect of proteases and heat

Proteases treated extracts showed a complete loss of activity, implying that the antimycobacterial activity may be due to the presence of peptides or protein (Fig. 5). Samples exposed to heat up to 70°C did not lose any activity however, there was a gradual decrease in the activity after 70°C, minimal activity at 100°C and complete loss at 121°C (Table 3). Control samples did not show any loss of antimycobacterial activity (Fig. 6a and 6b).

The sample showed MIC of 400 µg/ml with the indicator organism and DMSO with test media alone taken as control has shown luxuriant growth.

Bioprospecting plant associated microorganisms for potential bacteriocin like inhibitory substances (BLIS), in different settings and ecosystems is gaining significance to develop more effective and less toxic therapeutics (Ryan *et al.* 2008, Strobel *et al.* 2003). *F. benghalensis* belongs to Moraceae, Mulberry family has more than 800 species and 2000 varieties with remarkable medicinal value in ancient ethnomedicine and many studies have also confirmed their medicinal potency

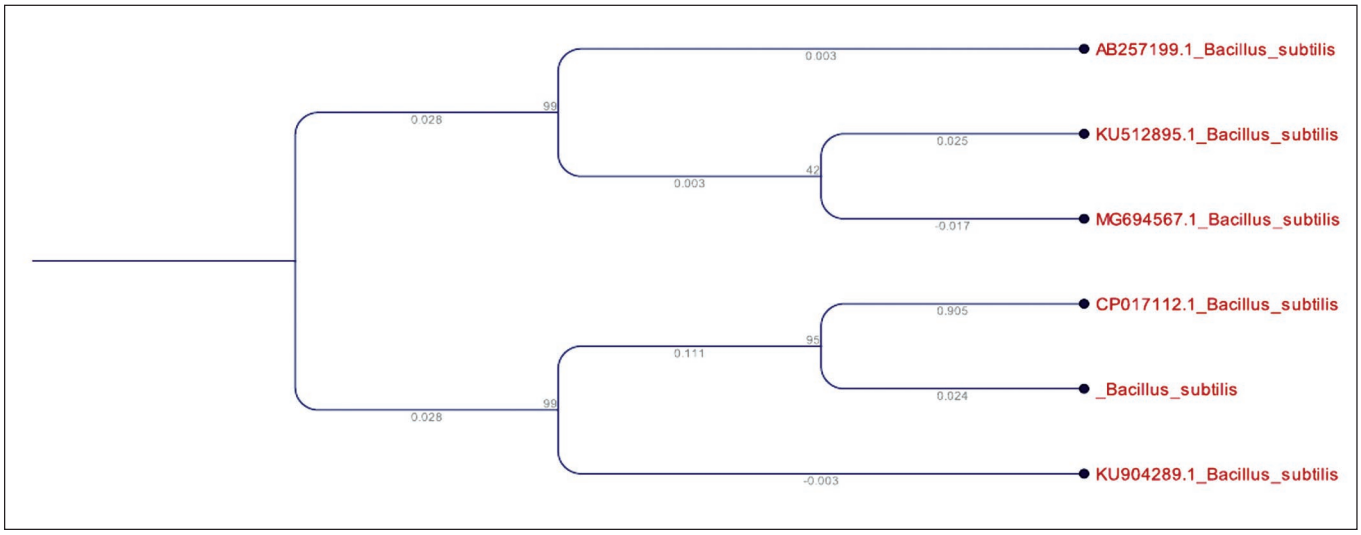

Fig. 3. Phylogenetic tree based on 16S rRNA sequences.

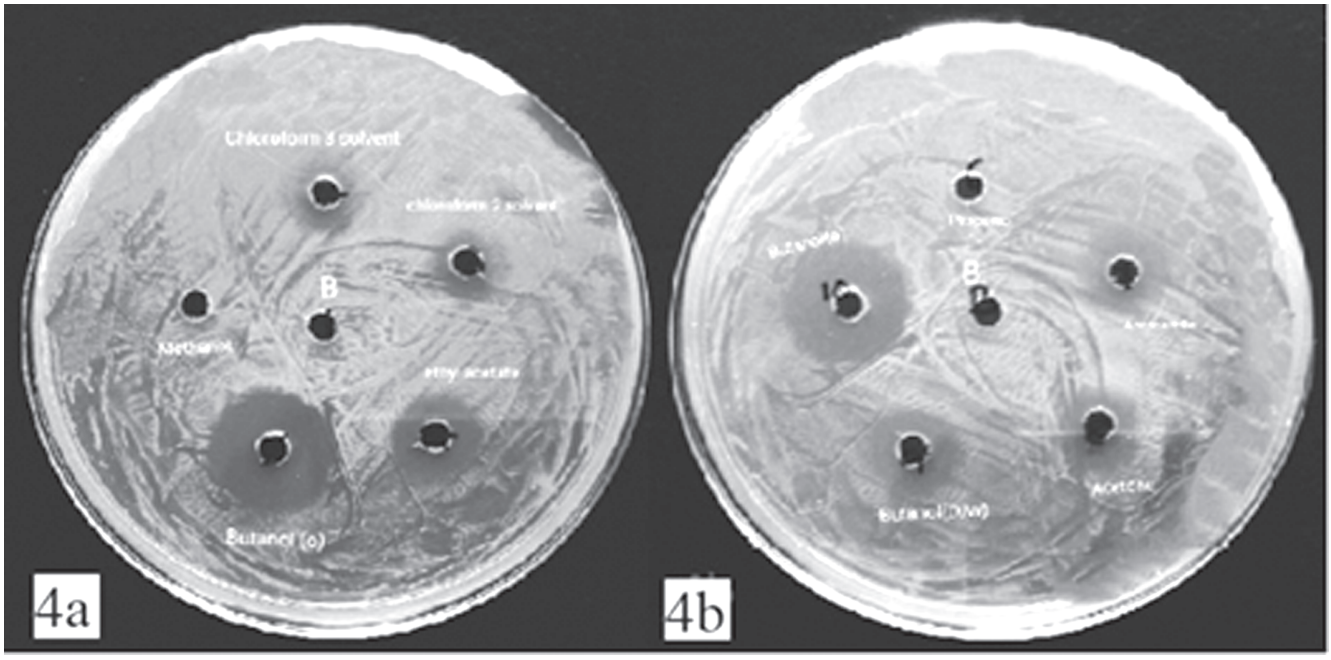

Fig. 4. (a and b): Antimycobacterial activity profile of isolate-1.

and antimicrobial activity (Tulasi *et al*. 2015).

Plant-bacteria associations are widely studied for antimicrobial activity, ubiquitous *Bacillus* species of soil and plant origin are well known to produce antimicrobial substances that can control pathogenic bacterial infections (Lim *et al*. 2016, Sumi *et al*. 2015). The present study is reporting for the first time, a *Bacillus subtilis* strain screened from the microbiome of *F. benghalensis* leaves that is capable of producing a potential antimycobacterial bacteriocin like inhibitory substance.

BLIS showed antimycobacterial activity when extracted with various organic solvents, indicating the presence of hydrophobic regions in BLIS, which are extractable with organic solvents which might be essential for antagonism. Studies reveal that the inactivation of micro-organisms by bacteriocins depends on the hydrophobic interaction between cells and bacteriocin molecules (Gao *et al*. 1991). The dried organic solvent extract dissolved in DMSO showed a maximum zone of inhibition than that in distilled water. DMSO, having polar

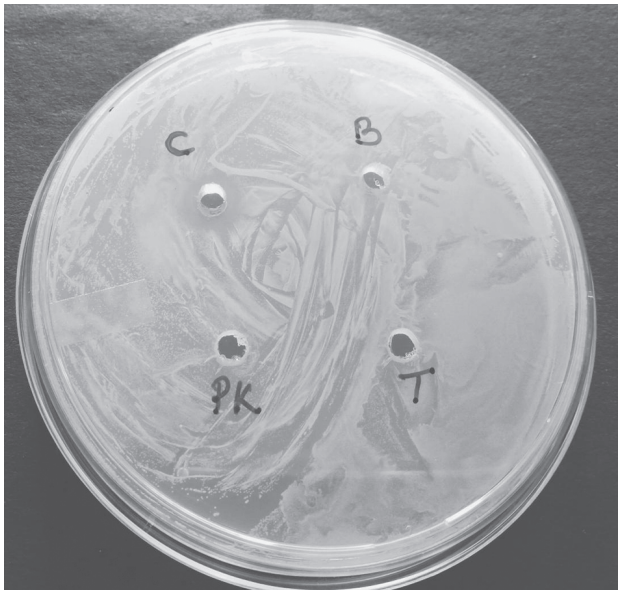

Fig. 5. Effect of Proteases,
[B – Blank (DMSO); T - Sample treated with Trypsin; PK - Sample treated with Proteinase K; C - Control (Protease untreated sample)].

aprotic nature, dissolves both polar and nonpolar compounds, revealing that the antimycobacterial activity is due to both polar and more of nonpolar compounds.

Proteinase K is a nonspecific proteolyzing enzyme which affects peptide bonds on target proteins at various sites, while trypsin acts on specific substrate sites. A complete loss of activity with these proteases confirms the proteinaceous nature of BLIS. Proteinaceous bacteriocins are considered as GRAS (generally recognized as safe) scale as they can be degraded by proteases in the human body, thus can act as natural food preservatives (Liu *et al.* 2015).

Heat stability of bacteriocins is an important property to be considered for bio-preservation, live-stock health, medicine and other applications (Bemena *et al.* 2014). The isolated BLIS retained 100% activity up to 70°C, which can be suitable for applications that do not require extensive heat treatments.

Antimicrobial peptides (AMP) are diverse group with novel mode of action and immunomodulatory activities. Added to their advantage, resistance to bacteriocins is proven tougher and infrequent for bacteria (Martínez *et al.* 2016). Cell selective toxicity and mechanism of action of AMPs are due to their charge, amphipathicity and hydrophobicity (Yeaman *et al*. 2003). The isolated BLIS has shown that the nonpolar biomolecules have potential activity supporting the characteristics of a Bacteriocin or an AMP that can combat resistant infections. Being organic solvent stable, proteinaceous in nature with significant heat stability and having a scope of betterment of current MIC by optimizing the BLIS producer, the isolated BLIS may have potential pharmacological applications.

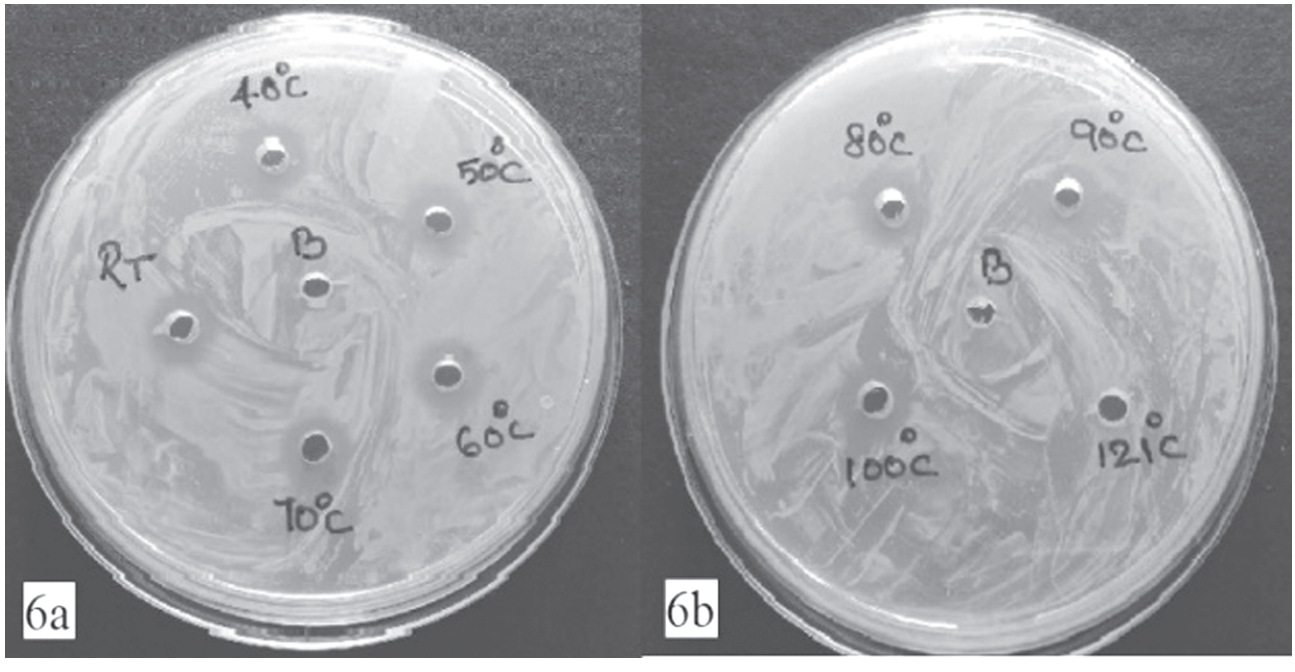

Fig. 6. Effect of heat on activity of BLIS for 10 min.
[(a) RT - 25°C, 40°C, 50°C, 60°C and 70°C; (b) 80°C, 90°C, 100°C and 121°C].

CONCLUSION

In recent years, though various approaches to treat bacterial infections have been developed, one promising alternative is antimicrobial peptides. Studies show that, AMPs can kill multiple bacterial species, inhibit developed biofilms, show strong synergy with several antibiotics, and act by targeting a universal stress response in bacteria. The BLIS isolated in the present study, revealed many promising characteristics which justifies its potentiality to combat MDR microbes as a precursor for the development of anti-mycobacterial drugs. Further optimizing the production and purification of BLIS can improve yield and efficacy for other pharmacological applications.

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